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Rehan, Shahid

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Functional reconstitution of human equilibrative nucleoside transporter-1 into styrene maleic acid co-polymer lipid particles

Shahid Rehan [a], Ville O. Paavilainen [b], Veli-Pekka Jaakola [a, *]

[a] Oulu Biocenter and Faculty of Biochemistry and Molecular Medicine, University of Oulu, P.O.Box 3000, FI-90014, Oulu, Finland
[b] Institute of Biotechnology, University of Helsinki, 00014 Helsinki, Finland

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ABSTRACT

The human equilibrative nucleoside transporter-1 (hENT1) is important for the entry of anti-cancer and anti-viral nucleoside analog therapeutics into the cell, and thus for their efficacy. Understanding of hENT1 structure-function relationship could assist with development of nucleoside analogs with better cellular uptake properties. However, structural and biophysical studies of hENT1 remain challenging as the hydrophobic nature of the protein leads to complete aggregation upon detergent-based membrane isolation. Here we report detergent-free reconstitution of the hENT1 transporter into styrene maleic acid co-polymer lipid particles (SMALPs) that form a native lipid disc. SMALP-purified hENT1, expressed in Sf9 insect cells binds a variety of ligands with a similar affinity as the protein in native membrane, and exhibits increased thermal stability compared to detergent-solubilized hENT1. hENT1-SMALPs purified using FLAG affinity M2 resin yielded ~ 0.4 mg of active and homogenous protein per liter of culture as demonstrated by ligand binding, size-exclusion chromatography and SDS-PAGE analyses. Electrospray ionization mass spectrometry (ESI-MS) analysis showed that each hENT1 lipid disc contains 16 phosphatidylcholine (PC) and 2 phosphatidylethanolamine (PE) lipid molecules. Polyunsaturated lipids are specifically excluded from the hENT1 lipid discs, possibly reflecting a functional requirement for a dynamic lipid environment. Our work demonstrates that human nucleoside transporters can be extracted and purified without removal from their native lipid environment, opening up a wide range of possibilities for their biophysical and structural studies.

1. Introduction

Nearly 20–30% of the human genome encodes for integral membrane proteins (IMPs), a class of proteins that require lipid membranes to maintain their structure and function. IMPs include ion channels, membrane-bound receptors, and transporters, with many IMPs acting as important therapeutic targets—approximately 50% of all commercially available drugs directly or indirectly target IMPs [1]. Nucleoside transporters are a physiologically important subset of IMPs that mediate transport of nucleosides involved in signaling [2,3] and cellular DNA/RNA synthesis [4]. Nucleoside transporters are also involved in cellular uptake of anti-cancer and anti-viral nucleoside analog therapeutics [5,6]. Mammalian cells contain two families of nucleoside transporters—SLC28 and SLC29—with different nucleoside binding specificities [4,7]. Human equilibrative nucleoside transporter-1 (hENT1) is a member of the SLC29 family and has been shown to transport adenosine, uridine, and thymidine. hENT1 also plays a critical role in the transport of several different chemotherapies such as gemcitabine and cisplatin [5,8]. Expression level of hENT1 is regulated to match the metabolic need of nucleosides uptake in proliferating cells, and it serves as a biomarkers for certain types of cancers [9,10].

Gaining an understanding of the structure and function of hENT1 is of significant interest both for understanding its diverse physiological functions and pathological functions as well as for drug discovery efforts—understanding how nucleoside analogs interact with hENT1 could facilitate development of compound with better uptake proper-

Abbreviations: hENT1, human equilibrative nucleoside transporter-1; NBMPR, S-(4-Nitrobenzyl)-6-thioinosine; dpm, disintegrations per minute; SMALPs, styrene maleic acid copolymer lipid particles..

* Corresponding author at: Center for Proteomic Chemistry, Novartis Institute for Biomedical Research, Virchov 16-2.249.04, CH-4056 Basel, Switzerland.
Email address: veli-pekka.jaakola@novartis.com (V-P Jaakola)

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ties. However, IMP purification in functional form remains one of the biggest challenges in studying membrane protein structure and function. These studies are often hampered by the hydrophobic nature of IMPs, which leads to aggregation upon isolation from lipid bilayers. Traditional methods of membrane protein purification use detergents or amphipols for extraction from lipid membranes [11,12]. However, removal from the native lipid environment can be destabilizing, leading to the loss of physiological properties and eventual protein aggregation. In addition, extended incubation in detergents can induce aggregation caused by the presence of low concentrations of free detergent monomers that stabilize more open, aggregation-prone protein conformations [13]. Attempts to add back lipids such as through reconstitution into liposomes or lipid bilayers may increase protein stability but these processes are labor-intensive and time consuming [14]. Furthermore, due to their large size and light scattering properties liposomes are incompatible with many modern biophysical techniques. For some IMPs, reconstitution into membrane scaffold protein (MSP)-containing nanodiscs has enabled successful structural and functional characterization free from the complications of detergent micelles and free detergent species [15]. However, such studies require detergent screening, solubilization, and purification prior to reconstitution, with additional optimizations such as choice of MSP protein, lipid-to-protein ratio, and method of detergent removal to initiate reconstitution [16].

A novel method for membrane solubilization and IMP encapsulation was recently described [17]. This method is based on the use of a chemically stable, biocompatible, and readily available styrene maleic acid copolymer (SMA). Active SMA is prepared by hydrolysis of the anhydrated SMA polymer under acidic conditions [18]. Two commercially available variants of SMA contain different ratios of styrene to maleic acid (2:1 or 3:1 ratios), and can differ in their ability to solubilize particular membrane proteins [17]. SMA is a highly amphipathic molecule that can self-assemble into disc-like structures of 10–12 nm in diameter, and capture membrane proteins within a native lipid environment [17,19,20]. The resulting SMALPs (also called native nanodiscs or lipid discs) are fully water-soluble assemblies that contain IMPs together with their respective native lipids [21]. SMALPs are emerging as a substitute for detergents in biophysical and structural characterization of membrane proteins that are prone to lose their native fold in complex detergent micelles [22]. Furthermore, recent advances in crystal-free structural characterization of membrane proteins using cryo-EM single particle methods [23–25] have opened new possibilities for studying SMALP-encapsulated IMP complexes.

Inspired by these studies we have evaluated the potential of SMA technology for functional reconstitution of the hENT1 transporter. Here, we report a simple protocol for detergent-free solubilization, and purification of hENT1 embedded in SMALPs. We demonstrate that the hENT1-SMALPs can be purified in a homogenous, stable form and bind hENT1 ligands with comparable affinities to the transporter in Sf9 membranes. Thus, hENT1-SMALPs provide a powerful tool for future high content chemical screens to identify and biophysically characterize new hENT1 inhibitors. Furthermore, hENT1-SMALPs will likely be suitable for structural studies into structure-function relationships using single particle cryo-EM approaches.

2. Materials and methods

2.1. Materials

All restriction enzymes and Phusion DNA polymerase were purchased from New England Biolabs, USA. Anti-FLAG M2 affinity resin, SLC29A1 antibody produced in rabbit (HPA012384) and asolectin were purchased from Sigma, USA. Goat anti-rabbit IgG-horseradish peroxidase (RPN4301) and PVDF membrane were purchased from Amersham™, UK. ECL Plus Western detection reagents were from Thermo Fisher, USA. Insect cell culture media was from Lonza, Switzerland. S-(4-Nitrobenzyl)-6-thioinosine (NBMPR), dilazep and dipyridamole were purchased from Tocris Bioscience, UK. Tritium-labeled NBMPR ([^{3}H]NBMPR) with a specific activity of 8.43 Ci/mmol was purchased from Moravek Biochemicals, USA. GF/B glass filter 96-well plates were purchased from Millipore, USA. SMA polymer (XIRAN® SZ30010) was from Polyscope Polymers, Netherlands.

2.2. Preparation of styrene-maleic acid copolymer (SMA)

Pre-hydrated poly (styrene-*co*-maleic) at a ratio of 2:1 (SZ30010, Mol. wt 10,000 g/mol, maleic context approx. 31% wt) was provided by Polyscope (Netherlands) as a 25% (*w/v*) sodium salt solution. Polymer was precipitated with concentrated HCl, washed 5 times with 100 mM HCl, and lyophilized overnight. A stock solution of SMAs (6%, *w/v*) was made in a buffer containing 10 mM HEPES, pH 8.0, and stored at − 20 °C. Hydrolysis was confirmed by FTIR analysis as descried previously [26].

2.3. Cloning and baculovirus expression of hENT1

hENT1 cDNA (NCBI gene ID: 2030) was PCR amplified using hENT1-specific primers containing sites for *Sph* I and *Kpn*I restriction enzymes, and cloned into a pFastBac1 vector. All constructs were sequence verified.

Insect cell expression was carried out as described previously [27]. Briefly, the pFastBac1 vector containing the full-length hENT1 gene was transformed into competent DH10Bac cells and plated out onto LB plates containing 10 μg/ml Tetracycline, 50 μg/ml kanamycin, 7.5 μg/ml gentamycin, and 40 μg/ml Blue-gal, and incubated for two days at 37 °C. The recombinant bacmid DNA was extracted using standard methods and used to co-transfect insect cells using Invitrogen's protocol. Recombinant baculovirus containing hENT1 was passaged three times to generate a high titer virus stock. Sf9 cells at density of 1.5×10^6 per ml were virally transduced with an MOI of 2 and protein expression was achieved by incubating the cells at 27 °C for two days. Cells were collected by centrifugation at 1000*g* for 5 min and disrupted in lysis buffer (50 mM HEPES, pH 8.0, 500 mM NaCl, 5 mM EDTA, and 1 mM PMSF) by dounce homogenization. Membranes were prepared using method described previously [27]. Briefly, unbroken cells were separated by centrifugation at 1000*g* for 10 min and membranes were isolated by ultracentrifugation at 235,000*g* for 1 h. Isolated membranes were resuspended in ice-cold buffer (50 mM HEPES, pH 8.0, 500 mM NaCl) and washed 3- to 4-times. Finally membranes were resuspended in storage buffer (50 mM HEPES, pH 8.0, 500 mM NaCl and 40% glycerol) and stored at − 70 °C until further analysis.

2.4. Solubilization of hENT1expressed in Sf9 membranes

hENT1-expressing Sf9 membranes were diluted to a final protein concentration of 3 mg/ml in buffer containing 50 mM HEPES, pH 8.0, 500 mM NaCl, and 10% glycerol, and then solubilized with 0.25% SMA in the presence of 0.2% cholesteryl hemisuccinate CHS (*w/v*) at °C for 20 h. Insoluble protein was separated by untracentrifugation at 235,000*g* for 1 h, and supernatant containing SMALP-reconstituted hENT1 was collected for further analysis. Detergent solubilization was carried out as described previously [27].

2.5. Preparation of crude liposomes

Proteoliposomes containing functional hENT1 were prepared according to the methods of Vickers et al. [28] with the following modifications. Sf9 membranes containing overexpressed hENT1 were solubi-

lized with 2% n-octyl-β-D-glucoside (β-OG) in a solubilization buffer (50 mM HEPES, pH 8.0, 500 mM NaCl, and 10% glycerol) for 2 h at 4 °C, followed by ultracentrifugation at 235,000*g* for 1 h to separate the insoluble membrane fraction. Solubilized hENT1 was then supplemented with asolectin and CHS solubilized with 2% β-OG at a molar ratio of 60:30:10, and incubated on ice for 30 min. Removal of the detergent and formation of crude liposomes was achieved by first diluting the reaction mixture below the CMC of β-OG (0.5%) and then completely removing detergent by dialyzing overnight at 4 °C Samples were ultracentrifuged at 235,000*g* for 1 h to collect the concentrated liposomes, which were flash frozen in liquid nitrogen, and stored at − 70 °C until further analysis.

2.6. Saturation and competition binding experiments

The saturation binding experiments were performed as described previously [27]. Briefly, 5–10 μg of isolated membrane, proteoliposome, and either SMA-solubilized or purified hENT1-SMALPs were diluted in transport buffer (10 mM Tris-HCl, pH 8.0) and incubated with increasing concentrations of [^{3}H]NBMPR, ranging from 0.125–30 nM, for 40 min at 22 °C. In order to assess non-specific ligand binding, identical reactions were prepared in the presence or absence of 10 μM unlabeled NBMPR or dipyridamole. Unbound ligand was removed by washing the reaction mixture with ice-cold transport buffer on 0.2 μm 96-well filter plates (Millipore) under vacuum. Plates were dried at room temperature and radioactivity was measured using a MicroBeta TriLux scintillation counter (Millipore). The amount of [^{3}H]NBMPR specifically bound to hENT1 was calculated as the difference between the amount of [^{3}H]NBMPR that bound in the presence and absence of 10 μM unlabeled NBMPR or dipyridamole. The dissociation constant K_d and B_{max} were calculated by fitting the experimental data to one site-specific binding equation using non-linear regression with Graphpad prism software.

For competition binding assays, protein was incubated with increasing concentrations of either unlabeled NBMPR, dipyridamole or dilazep for 10 min before adding 2.5 nM [^{3}H]NBMPR. Apparent inhibition constants (K_i values) were calculated using the Cheng and Prusoff equation [29].

$$K_i = IC_{50}/\left(1 + \left([L]/K_d\right)\right)$$

Where K_i is the equilibrium dissociation constant for unlabeled inhibitor, IC50 is the concentration causing 50% inhibition; L is the concentration of the radioligand, and K_d is the equilibrium dissociation constant for the radioligand.

2.7. Analysis of hENT1 stability in SMALPs

Stability of hENT1 in SMALPs was monitored using a radioligand-binding assay as described previously [27,30]. Briefly, 100 μl of hENT1 samples solubilized with either 0.25% (*w/v*) SMA or 1% of detergent (n-dodecyl-β-D-maltopyranoside (DDM) and decyl-β-DM) were incubated at temperatures ranging from 4 to 80 °C in 5 °C increments in a gradient thermocycler for 30 min. Control samples were kept on ice. After heating, samples were chilled on ice for 5 min and centrifuged at 235,000*g* for 30 min at 4 °C. Sf9 membrane samples were processed without centrifugation. [^{3}H]NBMPR binding activities were then measured by normalizing the data with the control sample (from 4 °C). Melting temperatures were calculated by fitting the experimental data to a non-linear Boltzmann sigmoidal equation using GraphPad Prism software.

2.8. Purification and characterization of SMALP-reconstituted hENT1

Purification of hENT1-SMALPs was carried out from Sf9 membrane isolated from 1 L of cell culture. Solubilization was carried out essentially as described above. The soluble fraction was collected and bound to 1 ml of pre-equilibrated FLAG M2 affinity resin overnight at 4 °C with mild agitation. FLAG M2 affinity resin with bound hENT1-SMALPs was loaded onto an empty Bio-Rad chromatography column, and the resin was then washed with 10–20 column volumes of wash buffer (50 mM HEPES, pH 8.0, 500 mM NaCl, and 5% glycerol). Finally, hENT1 was eluted with 100 μg/ml of FLAG peptide in elution buffer (20 mM HEPES pH 8.0, 500 mM NaCl and 5% glycerol). Concentration of hENT1 was determined spectrophotometrically using an extinction coefficient of 57,005 M^{-1} cm^{-1} at 280 nm. Final yield of purified hENT1 was quantified as 0.4 mg per liter of Sf9 culture.

Purified hENT1-SMALP protein was separated using 12% SDS-PAGE and visualized by both Coomassie Blue staining and immunoblotting using anti-His antibody and anti-SLC29A1 antibodies. Homogeneity of the purified protein was assessed by size-exclusion chromatography analysis. Briefly, purified protein was injected into Enrich SEC 650 10 × 300 column (BioRad) using ÄKTAPurifier system (GE health). Functional integrity of the protein was analyzed using a radioligand-binding assay as described above.

2.9. Lipid analysis

Identification and quantification of hENT1-SMALP lipids was carried out by ESI-MS analysis. Briefly, lipids from SMA-solubilized total membranes and purified hENT1-SMALPs were extracted according to the method as described earlier [31] and dissolved in chloroform:methanol (1:2 *v/v*). Immediately prior to mass spectrometry, NH_4OH was added to a final concentration of 1% to facilitate ionization. Aliquots were infused into the electrospray source of a triple quadrupole mass spectrometer (Agilent 6490 Triple Quad LC/MS with iFunnel Technology; Agilent Technologies, Santa Clara, CA) at flow rate of 10 μl/min, and mass spectra were recorded using both positive and negative ionization modes, and several lipid class-specific MS/MS scanning modes [32]. Mass spectra were processed with the MassHunter software (Agilent Technologies, Inc. California, USA), and the procedures of the quantitative data analysis utilizing LIMSA software as described previously [33]. Phosphatidylcholines (PCs, detected as precursors of *m/z* 184, P184) and phosphatidylethanolamines (PEs, detected as neutral loss of 141 mass units, NL141) species could be detected both in sample prepared from the purified protein and in the isolated membranes. To support identification of the acyl chain assembly of the lipid species isolated with the hENT1 protein, the fatty acid composition of the preparation was determined by gas chromatography essentially as detailed in [34].

3. Results and discussion

3.1. SMA solubilization of hENT1

To test the ability of SMA to solubilize functional hENT1, we used SMA to extract hENT1 from Sf9 cells overexpressing the protein. Our initial experiments indicated that SMA is capable of efficiently extracting hENT1 from Sf9 membranes, but that solubilization at room temperature resulted in significant protein degradation (Supplementary Fig. S1a). Therefore, we carried out all subsequent solubilization experiments at 4°C for overnight (12- to 18-hours) using 0.25% SMA, which resulted in approximately 20% solubilization efficiency (data not shown). We also discovered that while SMA extraction at concentra-

tions in excess of 0.5% (*w/v*), resulted in efficient hENT1 extraction, the solubilized transporter completely lacked ability to bind [^{3}H]NBMPR (Supplementary Fig. S1b). This was unexpected as many previous studies reported the use of 2–3% SMA for solubilizing diverse IMPs in functional form at room temperature followed by affinity purification [17,35]. We propose that at higher concentrations either the amphipathic SMA polymer interacts with the hydrophobic NBMPR ligand, or the highly positively charged carboxyl group of the SMA polymer interacts with charged groups on the hENT1 surface, disrupting the structure and rendering hENT1 non-functional.

In an effort to preserve the hENT1functionality, we added CHS and asolectin to SMA extraction, as our earlier study showed that these lipids stabilize detergent-solubilized hENT1 [27]. Here we found that addition of CHS and asolectin did not increase the solubilization efficiency, possibly due to competition between these lipids and native membrane lipids for solubilization by SMA. However, addition of both CHS and asolectin improved the ability of hENT1 to bind [^{3}H]NBMPR when solubilized with 0.25% (*w/v*) SMA (Supplementary Fig. S1a). It is unclear whether CHS and asolectin affect SMA-solubilized hENT1 function by directly interacting with hENT1, or if these lipids "chemically tune" the SMALP assembly process by increasing protein thermal stability. Cholesterol is known to enhance the lipid bilayer packing by occupying grooves and cavities formed by kinks in unsaturated fatty acid tails [36] and may stabilize IMPs in a similar way. In all subsequent experiments we used 0.25% (*w/v*) SMA supplemented with 0.2% CHS (*w/v*) for hENT1 solubilization.

3.2. hENT1-SMALP inhibitor binding

To examine whether SMALPs are capable of encapsulating hENT1 in a functional state indicative of native folding we carried out saturation binding experiments of SMA-solubilized hENT1 with [^{3}H]NBMPR (Fig. 1a, Supplementary Fig. S3A). These studies, carried out in the presence and absence of a large excess of pyridamole to control for non-specific [^{3}H]NBMPR binding, demonstrated saturable [^{3}H] NBMPR binding with a K_d of 3.0 ± 0.4 nM, a value comparable to earlier studies (1.2 ± 0.2 nM) using hENT1 expressed in Sf9 insect cells [27]. Similarly, hENT1-SMALPs retained affinity for the structurally unrelated inhibitors dilazep and dipyridamole. Consistent with our previous study [37] we observed monophasic behavior of these inhibitors in [^{3}H]NBMPR competition binding experiments where we obtained K_i values of 4.0 ± 0.4 nM, 34.0 ± 1.0 nM, and 527.4 ± 3.0 nM for unlabeled NBMPR, dilazep, and dipyridamole, respectively (Fig. 1b). These inhibitor constants are 4- to 33-fold higher than previously published values obtained using membrane vesicles and reconstituted proteoliposomes (Table 1). The increase in K_d and K_i may reflect small structural re-arrangements during solubilization and lipid disc formation. [^{3}H]NBMPR binding experiments with empty SMA (without hENT1) demonstrated only negligible binding, further suggesting a slight decrease in specific hENT1 ligand binding activity (Supplementary Fig. S3b). Another possible explanation is that the highly negatively charged SMA carboxylic groups may promote hENT1 to adopt a non-physiological protein conformation at higher SMA concentrations.

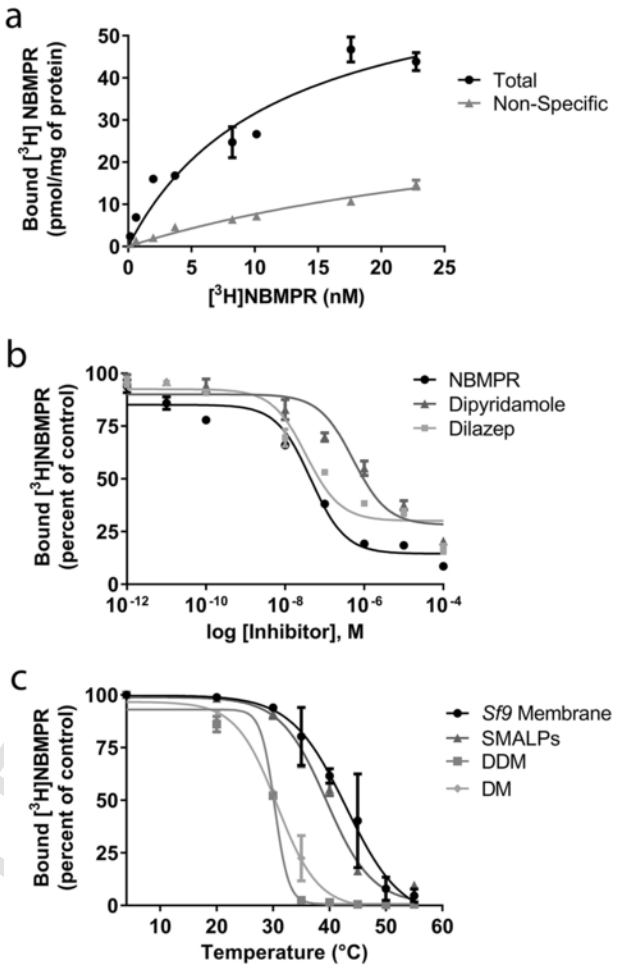

Fig. 1. Functional and thermostability analysis of hENT1-SMALPs. (a) Equilibrium binding of [^{3}H]NBMPR (0.12 nM–30 nM) to SMA-solubilized hENT1 transporter. Results are presented as the amount of specifically bound [^{3}H]NBMPR (after subtraction of non-specifically bound fraction) as a function of free [^{3}H]NBMPR. Each data point represents an average of at least three measurements. Three experiments gave similar results yielding a K_d of 3.0 ± 0.4 nM. (b) [^{3}H]NBMPR competition binding by different inhibitors. SMA-solubilized hENT1 incubated with 3.5 nM [^{3}H] NBMPR alone or together with either unlabeled NBMPR, dilazep or dipyridamole over a concentration range of 0.01 pM to 100 μM. Results are shown as the percentage of [^{3}H] NBMPR bound as a function of the logarithm of the concentration of unlabeled competitor. The amount of [^{3}H]NBMPR that bound in the absence of inhibitors was taken as 100% binding. Each point is the average of at least three measurements. Two independent experiments showed similar results yielding K_i values of 4.0 ± 0.4 nM, 34.0 ± 1.0 nM, and 527 ± 3 nM for NBMPR, dilazep and dipyridamole, respectively. (c) Thermostability analysis of hENT1 in different environments. The protein was solubilized with either SMALPs or detergent and heated in a thermocycler for 30 min at 4 °C–80 °C and [^{3}H]NBMPR binding activities were subsequently measured as described in the methods section. All experiments were repeated at least three times. Each data point is the average of at least three measurements.

Table 1
Pharmacological characterization of hENT1 in different membrane systems.

Inhibitor/drug [K_i (nM)]	SMALP[2]	*Sf9* membrane[a]	Proteoliposome[b]	LMNG[a]	DM[a]
[^{3}H]NBMPR	4.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.3	7.0 ± 2.0	10.0 ± 0.5
Dilazep	34.0 ± 1.0	3.0 ± 0.1	4.0 ± 0.5	37.0 ± 1.0	–
Dipyridamole	527.4 ± 3.0	16.0 ± 1.0	33.0 ± 3.3	303.0 ± 1.0	–

[a] Rehan, S and Jaakola, V.P. [27].
[b] Current study.

Nevertheless, the rank order of hENT1 affinities between the tested inhibitors is consistent with all previous studies [28,37,38], suggesting that the overall activity of hENT1 in the SMALP platform is comparable to physiological activity, and that SMALPs can be used for further biophysical investigations of hENT1 and possibly other nucleoside transporters.

When compared to reconstitution in detergents, hENT1-SMALPs perform similarly. hENT1 reconstituted in SMALPs retained pharmacological properties similar to those in lauryl maltose neopentyl glycol (LMNG) and DM detergents, although the DM-solubilized hENT1 had nearly 4-fold low binding affinity for NBMPR compared to membrane vesicles (Table 1, Supplementary Fig. S1, c-d). hENT1 reconstitution in SMALPs is more efficient than solubilization in DM detergent. However, given the potential interference of detergents with many biophysical techniques, detergent-induced destabilization of membrane proteins and the difficulties in removing detergents with very low CMCs, the SMALP platform becomes an attractive choice for solubilizing hENT1 for functional studies.

3.3. hENT1-SMALP thermostability

To further verify the correct folding of SMALP-embedded hENT1, we investigated the thermal stability of hENT1-SMALPs using the above-described radioligand-binding assay [22]. The temperature dependent decrease in the ability of hENT1-SMALPs to bind [^{3}H]NBMPR was used to indicate the loss of correct folding. The obtained binding data at different temperatures was fitted to a sigmoid curve describing the thermotrophic transition from folded to the unfolded or aggregated form of the protein. From this plot, we defined an apparent melting temperature (T_m) as the temperature at which radioligand-binding activity dropped to 50% of maximum. This analysis revealed an apparent T_m of 45 °C for the hENT1 transporter in Sf9 membranes (Fig. 1c). Solubilization with either DDM or DM resulted in a marked decrease in thermostability (T_m of 30 °C and 31 °C, respectively). However, the SMA-solubilized hENT1 retained thermal stability closer to the apparent melting temperature in Sf9 cell membranes (T_m of 37 °C and 45 °C, respectively.

A common problem associated with detergent solubilization of membrane proteins is destabilization of native protein structure in the solubilized state, which is likely caused by varying degrees of delipidation [12,39]. In the case of SMALPs, annular lipids become encapsulated and are retained during assembly, thereby decreasing the likelihood of activity loss due to lipid depletion [40,41]. This effect was clearly observed when comparing hENT1-SMALPs with detergent-solubilized hENT1 (Fig. 1c). Yet, an even greater degree of thermostabilization was achieved by solubilization with LMNG [27]. However, while this appears to suggest that LMNG is a better choice for hENT1 characterization, it should be noted that LMNG forms complex micelles, and due to their extremely low critical micelle concentration (CMC ~ 0.0001%), it is practically impossible to completely remove LMNG using conventional detergent removal methods [13]. These micelle properties are known to interfere with IMP characterization by biophysical and structural methods such as vapor-diffusion-based crystallization, single particle cryo-EM and NMR [12,13,42]. Therefore, a significant increase in thermal stability compared to maltoside detergents makes SMALPs an excellent choice for future structural studies on hENT1.

3.4. Analysis of purified hENT1 in SMALPs

Purification of hENT1-SMALPs was carried out from total cellular membranes prepared from a 1 l culture of Sf9 cells infected with a recombinant baculovirus expressing 8xHis-FLAG tagged hENT1 [27]. Following SMA-solubilization and affinity purification with FLAG M2 resin, (Supplementary Fig. S2a) the purified protein yield was estimated as ~ 0.5 mg per liter of culture (Table 2). On SDS-PAGE, the purified protein runs as a major band at the expected molecular weight of 45 kDa with minor multimeric SDS-resistant species observed at higher molecular weights (Fig. 2a and Supplementary Fig. S2a-b). Western blot analysis with an isoform-specific antibody was used to verify that these observed bands correspond to intact hENT1. The presence of a C-terminal 8xHis tag was confirmed by probing with an anti-His antibody, whereas the N-terminal FLAG tag was used for affinity purification (Supplementary Fig. S2b). Size-exclusion chromatography analysis on an Enrich SEC 650 10 × 300 column (BioRad) indicated that the monomeric hENT1-SMALPs are homogenous and elute at approximately 130–140 kDa molecular weight (Fig. 2a, Supplementary Fig. S2c-d). We then analyzed the functional integrity of purified hENT1-SMALPs by [^{3}H]NBMPR binding assay, which revealed no significant loss of activity during purification (K_d of 8.0 ± 2.0 nM and 3.4 ± 0.4 nM for purified and solubilized hENT1-SMALPs, respectively) (Fig. 2b). Bmax determination from this experiment revealed that 877 pmol/mg of hENT1 retains [^{3}H]NBMPR binding activity. This corresponds to approximately 92 ± 4% specific hENT1 activity in the final preparation (Table 2). Furthermore, single non-saturating concentration of [^{3}H]NBMPR used to estimated Bmax value of various fractions

Table 2
Yield and activity of hENT1-SMALPs during affinity purification steps.

Fraction	Yield (mg)	Purity (%)	% Activity
Sf9 membrane	4.5[a]	–	–
SMALPs-solubilized hENT1	1.0[a]	–	–
Purified hENT1-SMALPs	0.4[b]	> 90	92 ± 4%[c]

[a] Total protein estimated by BCA assay.
[b] hENT1-SMALPs protein was estimated using nanodrop spectrometer (280 nm).
[c] Estimated from the Bmax values of two independent experiments.

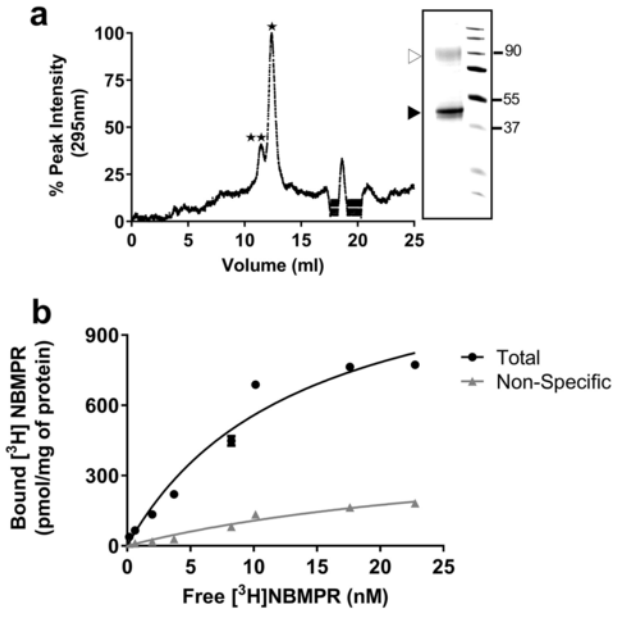

Fig. 2. Purification and characterization of hENT1-SMALPs. (a) Saturation binding assay of purified hENT1-SMALPs with [^{3}H]NBMPR using the method described in the main text. (b) Size exclusion chromatography analysis of purified hENT1-SMALPs on Enrich SEC 650 10 × 300 column (Bio-Rad) with a flow rate of 0.5 ml/min. The relative size of protein-SMALP complexes was estimated using elution profiles of protein standards detected by UV detector (see supplementary Fig. S2c-d). Inset: Purified hENT1-SMALPs monomer (*) and dimer (**) eluted during gel filtration. On SDS-PAGE monomer (►) near 45 kDa and dimer (▷) 90 kDa were detected with Coomassie blue staining.

that shows 8-fold enrichment of hENT1 during purification (Supplementary Fig. S3c).

3.5. Lipids in hENT1-SMALP assembly

The composition of the surrounding lipid bilayer is important for activity and stability of many IMPs [43–45]. We therefore, set out to establish the identity and stoichiometry of individual lipid species present in the annular hENT1 lipid bilayer. For this purpose, we performed lipid extraction from purified hENT1-SMALPs as well as from Sf9 membrane derived from cells infected with a control virus that does not express hENT1. ESI-MS/MS tandem mass spectrometry analysis revealed prominent suppression of many lipid peaks by several artifact peaks arising from the SMA polymer (data not shown), however, using a class specific scanning mode we were able to identify and quantitatively characterize the PC and PE lipids species that form the main constituents of the Sf9 cell membrane [46]. Quantitation of the phospholipid content of purified hENT1-SMALPs, using purified reference lipids, revealed that hENT1 co-purifies as a complex with approximately 16 and 2 associated PE lipids, respectively. These findings are in good correlation with previous reports on phospholipid inclusion with diverse purified IMP-SMALPs (Table S3) [17]. Interestingly, the purified hENT1-SMALPs exhibited selective enrichment of long chain PC species when compared to SMA-solubilized Sf9 cell membrane controls (Fig. 3a). In contrast, analysis of PE lipids species revealed complete exclusion of polyunsaturated lipid species that are detected in Sf9 membrane controls (Fig. 3b, Table S1). Furthermore, GC–MS analysis confirmed that purified hENT1-SMALPs only contain saturated and monounsaturated fatty acids (Table S2).

The physiological functions of many IMPs can be greatly influenced by the composition of membrane lipids; while annular lipids (the first lipid layer around an IMP) may non-specifically interact with IMPs, the non-annular lipids selectively bind and regulate IMP function and correct folding [47]. These lipids often co-purify with proteins and can withstand the excessive delipidation that removes most annular lipids during detergent solubilization. Under physiological conditions, protein conformational changes at protein-bilayer boundaries alter lipid packing which in turn is influenced by physiochemical properties of lipids and the degree of unsaturation in their fatty acyl chains [48]. Since polyunsaturated lipids are known to promote membrane rigidity and plasma membrane dynamics they may have important significance for the function of proteins with high degree of inherent conformational flexibility such as hENT1 [49].

4. Conclusion

In this paper we have explored the potential of SMA technology for the purification and characterization of nucleoside transporters. We have successfully purified the hENT1 transporter in a functional form with comparable ligand binding properties as previously reported for the transporter reconstituted in a lipid bilayer membrane. We found that purified hENT1-SMALPs are comparatively more stable than hENT1 solubilized in detergents such as DDM and DM and that the lipid composition of SMALP-hENT1 is highly enriched in PC lipids with saturated and monounsaturated fatty acids. Our data suggests that hENT1-SMALPs are an excellent platform for biophysical and structural characterization of the transporter.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2017.02.017.

Transparency document

The Transparency document associated with this article can be found, in the online version.

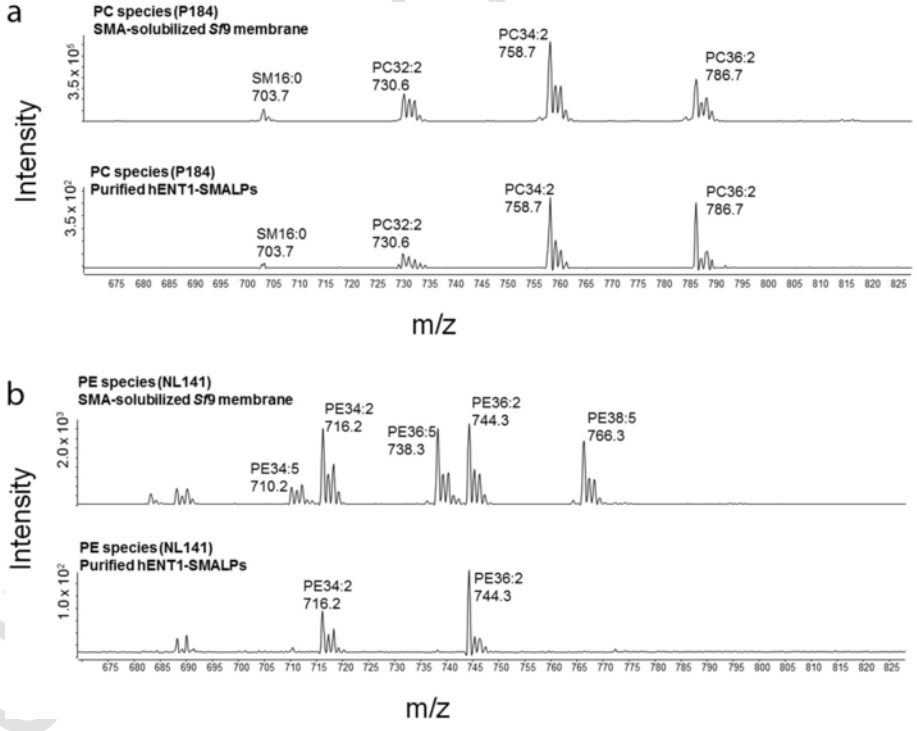

Fig. 3. Analysis of the lipid content of hENT1-SMALP particles. (a) (ESI-MS/MS analysis of the lipid content of SMALPs-solubilized Sf9 membranes and purified hENT1-SMALPs. Lipid analysis was carried out as described in the method section. (a) and (b) panels show PC and PE lipid enrichment in SMA-solubilized Sf9 cell membranes and purified hENT1-SMALPs, respectively.

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References

[1] J.P. Keogh, Membrane transporters in drug development, Adv. Pharmacol. 63 (2012) 1–42.
[2] A. Dos Santos-Rodrigues, M.R. Pereira, R. Brito, N.A. de Oliveira, R. Paes-de-Carvalho, Adenosine transporters and receptors: key elements for retinal function and neuroprotection, Vitam. Horm. 98 (2015) 487–523.
[3] S.W. Krauss, R.B. Ghirnikar, I. Diamond, A.S. Gordon, Inhibition of adenosine uptake by ethanol is specific for one class of nucleoside transporters, Mol. Pharmacol. 44 (1993) 1021–1026.
[4] S.A. Baldwin, P.R. Beal, S.Y. Yao, A.E. King, C.E. Cass, J.D. Young, The equilibrative nucleoside transporter family, SLC29, Pflugers Arch. 447 (2004) 735–743.
[5] J.R. Mackey, S.Y. Yao, K.M. Smith, E. Karpinski, S.A. Baldwin, C.E. Cass, J.D. Young, Gemcitabine transport in xenopus oocytes expressing recombinant plasma membrane mammalian nucleoside transporters, J. Natl. Cancer Inst. 91 (1999) 1876–1881.
[6] C.A. Koczor, R.A. Torres, W. Lewis, The role of transporters in the toxicity of nucleoside and nucleotide analogs, Expert Opin. Drug Metab. Toxicol. 8 (2012) 665–676.
[7] J.D. Young, S.Y. Yao, J.M. Baldwin, C.E. Cass, S.A. Baldwin, The human concentrative and equilibrative nucleoside transporter families, SLC28 and SLC29, Mol. Asp. Med. 34 (2013) 529–547.
[8] N. Matsumura, Y. Nakamura, Y. Kohjimoto, T. Inagaki, Y. Nanpo, H. Yasuoka, Y. Ohashi, I. Hara, The prognostic significance of human equilibrative nucleoside transporter 1 expression in patients with metastatic bladder cancer treated with gemcitabine-cisplatin-based combination chemotherapy, BJU Int. 108 (2011) E110–E116.
[9] W. Greenhalf, P. Ghaneh, J.P. Neoptolemos, D.H. Palmer, T.F. Cox, R.F. Lamb, E. Garner, F. Campbell, J.R. Mackey, E. Costello, M.J. Moore, J.W. Valle, A.C. McDonald, R. Carter, N.C. Tebbutt, D. Goldstein, J. Shannon, C. Dervenis, B. Glimelius, M. Deakin, R.M. Charnley, F. Lacaine, A.G. Scarfe, M.R. Middleton, A. Anthoney, C.M. Halloran, J. Mayerle, A. Olah, R. Jackson, C.L. Rawcliffe, A. Scarpa, C. Bassi, M.W. Buchler, Pancreatic cancer hENT1 expression and survival from gemcitabine in patients from the ESPAC-3 trial, J. Natl. Cancer Inst. 106 (2014) djt347.
[10] J. Lane, T.A. Martin, C. McGuigan, M.D. Mason, W.G. Jiang, The differential expression of hCNT1 and hENT1 i n breast cancer and the possible impact on breast cancer therapy, J. Exp. Ther. Oncol. 8 (2010) 203–210.
[11] C. Tribet, R. Audebert, J.L. Popot, Amphipols: polymers that keep membrane proteins soluble in aqueous solutions, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 15047–15050.
[12] A.M. Seddon, P. Curnow, P.J. Booth, Membrane proteins, lipids and detergents: not just a soap opera, Biochim. Biophys. Acta 1666 (2004) 105–117.
[13] F. Hauer, C. Gerle, N. Fischer, A. Oshima, K. Shinzawa-Itoh, S. Shimada, K. Yokoyama, Y. Fujiyoshi, H. Stark, GraDeR: membrane protein complex preparation for single-particle cryo-EM, Structure 23 (2015) 1769–1775.
[14] P. Simeonov, S. Werner, C. Haupt, M. Tanabe, K. Bacia, Membrane protein reconstitution into liposomes guided by dual-color fluorescence cross-correlation spectroscopy, Biophys. Chem. 184 (2013) 37–43.
[15] I.G. Denisov, S.G. Sligar, Nanodiscs for structural and functional studies of membrane proteins, Nat. Struct. Mol. Biol. 23 (2016) 481–486.
[16] J. Borch, T. Hamann, The nanodisc: a novel tool for membrane protein studies, Biol. Chem. 390 (2009) 805–814.
[17] J.M. Dorr, S. Scheidelaar, M.C. Koorengevel, J.J. Dominguez, M. Schafer, C.A. van Walree, J.A. Killian, The styrene-maleic acid copolymer: a versatile tool in membrane research, Eur. Biophys. J. 45 (2016) 3–21.
[18] S.C. Lee, T.J. Knowles, V.L. Postis, M. Jamshad, R.A. Parslow, Y.P. Lin, A. Goldman, P. Sridhar, M. Overduin, S.P. Muench, T.R. Dafforn, A method for detergent-free isolation of membrane proteins in their local lipid environment, Nat. Protoc. 11 (2016) 1149–1162.
[19] D.J. Swainsbury, S. Scheidelaar, R. van Grondelle, J.A. Killian, M.R. Jones, Bacterial reaction centers purified with styrene maleic acid copolymer retain native membrane functional properties and display enhanced stability, Angew. Chem. Int. Ed. Eng. 53 (2014) 11803–11807.
[20] M. Wheatley, J. Charlton, M. Jamshad, S.J. Routledge, S. Bailey, P.J. La-Borde, M.T. Azam, R.T. Logan, R.M. Bill, T.R. Dafforn, D.R. Poyner, GPCR-styrene maleic acid lipid particles (GPCR-SMALPs): their nature and potential, Biochem. Soc. Trans. 44 (2016) 619–623.
[21] T.J. Knowles, R. Finka, C. Smith, Y.P. Lin, T. Dafforn, M. Overduin, Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer, J. Am. Chem. Soc. 131 (2009) 7484–7485.
[22] M. Jamshad, J. Charlton, Y.P. Lin, S.J. Routledge, Z. Bawa, T.J. Knowles, M. Overduin, N. Dekker, T.R. Dafforn, R.M. Bill, D.R. Poyner, M. Wheatley, G-protein coupled receptor solubilization and purification for biophysical analysis and functional studies, in the total absence of detergent, Biosci. Rep. 35 (2015).
[23] M. Bokori-Brown, T.G. Martin, C.E. Naylor, A.K. Basak, R.W. Titball, C.G. Savva, Cryo-EM structure of lysenin pore elucidates membrane insertion by an aerolysin family protein, Nat. Commun. 7 (2016) 11293.
[24] K.N. Goldie, P. Abeyrathne, F. Kebbel, M. Chami, P. Ringler, H. Stahlberg, Cryo-electron microscopy of membrane proteins, Methods Mol. Biol. 1117 (2014) 325–341.
[25] V.A. Kostyuchenko, E.X. Lim, S. Zhang, G. Fibriansah, T.S. Ng, J.S. Ooi, J. Shi, S.M. Lok, Structure of the thermally stable Zika virus, Nature 533 (2016) 425–428.
[26] S. Banerjee, T.K. Pal, S.K. Guha, Probing molecular interactions of poly(styrene-co-maleic acid) with lipid matrix models to interpret the therapeutic potential of the co-polymer, Biochim. Biophys. Acta 1818 (2012) 537–550.
[27] S. Rehan, V.P. Jaakola, Expression, purification and functional characterization of human equilibrative nucleoside transporter subtype-1 (hENT1) protein from Sf9 insect cells, Protein Expr. Purif. 114 (2015) 99–107.
[28] M.F. Vickers, R.S. Mani, M. Sundaram, D.L. Hogue, J.D. Young, S.A. Baldwin, C.E. Cass, Functional production and reconstitution of the human equilibrative nucleoside transporter (hENT1) in Saccharomyces cerevisiae. Interaction of inhibitors of nucleoside transport with recombinant hENT1 and a glycosylation-defective derivative (hENT1/N48Q), Biochem. J. 339 (Pt 1) (1999) 21–32.
[29] Y. Cheng, W.H. Prusoff, Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50% inhibition (I50) of an enzymatic reaction, Biochem. Pharmacol. 22 (1973) 3099–3108.
[30] M.J. Serrano-Vega, F. Magnani, Y. Shibata, C.G. Tate, Conformational thermostabilization of the beta1-adrenergic receptor in a detergent-resistant form, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 877–882.
[31] J. Folch, M. Lees, G.H. Sloane Stanley, A simple method for the isolation and purification of total lipides from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[32] B. Brugger, G. Erben, R. Sandhoff, F.T. Wieland, W.D. Lehmann, Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 2339–2344.
[33] P. Haimi, A. Uphoff, M. Hermansson, P. Somerharju, Software tools for analysis of mass spectrometric lipidome data, Anal. Chem. 78 (2006) 8324–8331.
[34] K. Eder, Gas chromatographic analysis of fatty acid methyl esters, J. Chromatogr. B Biomed. Appl. 671 (1995) 113–131.
[35] S. Gulati, M. Jamshad, T.J. Knowles, K.A. Morrison, R. Downing, N. Cant, R. Collins, J.B. Koenderink, R.C. Ford, M. Overduin, I.D. Kerr, T.R. Dafforn, A.J. Rothnie, Detergent-free purification of ABC (ATP-binding-cassette) transporters, Biochem. J. 461 (2014) 269–278.
[36] J. Fantini, F.J. Barrantes, How cholesterol interacts with membrane proteins: an exploration of cholesterol-binding sites including CRAC, CARC, and tilted domains, Front. Physiol. 4 (2013) 31.
[37] S. Rehan, Y. Ashok, R. Nanekar, V.P. Jaakola, Thermodynamics and kinetics of inhibitor binding to human equilibrative nucleoside transporter subtype-1, Biochem. Pharmacol. 98 (2015) 681–689.
[38] S.Y. Yao, A.M. Ng, W.R. Muzyka, M. Griffiths, C.E. Cass, S.A. Baldwin, J.D. Young, Molecular cloning and functional characterization of nitrobenzylthioinosine (NBMPR)-sensitive (es) and NBMPR-insensitive (ei) equilibrative nucleoside transporter proteins (rENT1 and rENT2) from rat tissues, J. Biol. Chem. 272 (1997) 28423–28430.
[39] S. Kalipatnapu, A. Chattopadhyay, Membrane protein solubilization: recent advances and challenges in solubilization of serotonin1A receptors, IUBMB Life 57 (2005) 505–512.
[40] M. Jamshad, Y.P. Lin, T.J. Knowles, R.A. Parslow, C. Harris, M. Wheatley, D.R. Poyner, R.M. Bill, O.R. Thomas, M. Overduin, T.R. Dafforn, Surfactant-free purification of membrane proteins with intact native membrane environment, Biochem. Soc. Trans. 39 (2011) 813–818.
[41] M.C. Orwick, P.J. Judge, J. Procek, L. Lindholm, A. Graziadei, A. Engel, G. Grobner, A. Watts, Detergent-free formation and physicochemical characterization of nanosized lipid-polymer complexes: Lipodisq, Angew. Chem. Int. Ed. Eng. 51 (2012) 4653–4657.
[42] W. Liu, V. Cherezov, Crystallization of membrane proteins in lipidic mesophases, J. Vis. Exp. (**49**, 2011, e2501).
[43] H. Hong, Role of lipids in folding, misfolding and function of integral membrane proteins, Adv. Exp. Med. Biol. 855 (2015) 1–31.
[44] C. Lange, J.H. Nett, B.L. Trumpower, C. Hunte, Specific roles of protein-phospholipid interactions in the yeast cytochrome bc1 complex structure, EMBO J. 20 (2001) 6591–6600.
[45] J.T. Hopper, Y.T. Yu, D. Li, A. Raymond, M. Bostock, I. Liko, V. Mikhailov, A. Laganowsky, J.L. Benesch, M. Caffrey, D. Nietlispach, C.V. Robinson, Detergent-free mass spectrometry of membrane protein complexes, Nat. Methods 10 (2013) 1206–1208.

[46] K. Marheineke, S. Grunewald, W. Christie, H. Reilander, Lipid composition of Spodoptera frugiperda (Sf9) and Trichoplusia ni (Tn) insect cells used for baculovirus infection, FEBS Lett. 441 (1998) 49–52.
[47] P.V. Escriba, J.M. Gonzalez-Ros, F.M. Goni, P.K. Kinnunen, L. Vigh, L. Sanchez-Magraner, A.M. Fernandez, X. Busquets, I. Horvath, G. Barcelo-Coblijn, Membranes: a meeting point for lipids, proteins and therapies, J. Cell. Mol. Med. 12 (2008) 829–875.
[48] A.G. Lee, How lipids affect the activities of integral membrane proteins, Biochim. Biophys. Acta 1666 (2004) 62–87.
[49] S. Yazdi, M. Stein, F. Elinder, M. Andersson, E. Lindahl, The molecular basis of polyunsaturated fatty acid interactions with the shaker voltage-gated potassium channel, PLoS Comput. Biol. 12 (2016), e1004704.